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(54) Title: SEPARATION OF PROTEINS AND DYES

(57) Abstract

Problem: when proteins are purified using a protein-binding dye immobilised on a chromatographic matrix, the dye or a portion/derivative may leak into the eluant. Solution: an ion-exchange resin (e.g. Dowex-1) and a disrupting material (e.g. salt and a fatty acid such as sodium octanoate) are used to separate the dye from the protein.

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SEPARATION OF PROTEINS AND DYES

The present invention relates to the purification of proteins. In this specification, the term "protein" includes naturally-occurring proteins, non-naturally-occurring proteins and other polypeptides which are large enough to have a ligand binding site, and the term "purification" means "rendering more pure", rather than conferring a given level of purity.

In the separation of proteins from natural sources or, particularly, from the media of fermentations in which a genetically engineered host cell produces the protein, a protein-containing liquid is often passed through a chromatographic column consisting of a protein-binding compound bound to a solid support. The protein-binding compound binds to a ligand-binding site on the protein whilst the other material passes through the column and the protein is later eluted from the column in a purer form.

However, a small proportion of the protein-binding compound and/or a portion thereof sometimes elutes with the protein and must later be separated from the protein, particularly if the protein is intended for medical use. There have been prior proposals simply to absorb the dye onto a column of cross-linked Sephadex (R.T.M., Pharmacia).

Scopes, R.K., in "Protein Purification, Principles and

Practice" (Springer Verlag, N.Y., USA, 2nd Edition, pp 141-157), mentioned that trace amounts of dye in the eluate from dye-containing columns can be removed on anion exchangers but did not disclose whether it was the protein or the dye which should bind to the anion exchanger and did not mention the use of a disrupting agent. GB-A-2 053 296 disclosed the use of, amongst other things, a buffer containing sodium chloride and sodium caprylate to elute human serum albumin from an affinity medium. However, what those in the art would then have done, whether or not a dye contamination problem was perceived, was to dialyse away the salt and caprylate before further treatment. What we have now found is that combining the anion exchanger process with the use of a high salt/caprylate concentration to disrupt the dye-protein binding allows efficient separation of the dye from the desired protein.

Accordingly, one aspect of the present invention provides a process for removing some or all of a protein-binding compound from an aqueous liquid containing the protein-binding compound and a protein to which it can bind or is bound, the process comprising the steps of (1) exposing the liquid to a disrupting material to disrupt binding of the protein to the protein-binding material, (2) exposing the liquid to an ion exchange resin to bind the protein-binding material to the resin and (3) separating the resin from the liquid.

Steps (1) and (2) may be simultaneous or may at least overlap such that the liquid is still exposed to the disrupting agent at the time that it is exposed to the resin. Step (3) is usually performed by passing the liquid through a column of the resin such that a solution of the protein, relatively free of the protein-binding material, is obtained.

The process is particularly well suited to removing synthetic textile dye compounds of the sort which have been disclosed in the literature for purifying proteins. Many such proteins (probably thousands) can be purified by the use of such dyes. To pick just one dye, Cibacron Blue 3-GA, this can be used to purify kinases, dehydrogenases and most other enzymes requiring adenyl-containing co-factors, for example $NADP^+$ and NAD^+. Such proteins include alcohol dehydrogenase, adenylate cyclase, adenylate kinase, glucose-6-phosphate dehydrogenase, hexokinase, phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase. Although the Cibacron Blue 3-GA dye will bind to these classes of proteins, it is also possible to use the Cibacron Blue 3-GA dye to purify proteins that do not have the dinucleotide binding site. These include albumin, lipoproteins, blood coagulation factors, interferon and thyroxin binding globulin. These dye compounds are usually anionic, in which case an anion-exchanger is most appropriate in the process of the invention, but some are cationic, in which case a cation-

exchanger is most appropriate. The protein-binding compound is preferably a polysulphonated aromatic compound and is most preferably a triazine dye. Procion Brown MX-5BR, Cibacron Blue 3-GA, (suitable for separating human serum albumin), Procion Red H-8BN (for carboxypeptidase G2), Procion Yellow MX-AG (for IMP dehydrogenase), Procion Red HE-3B (for lactate dehydrogenase), Procion Green H-4G (for hexokinase), Procion Blue MX-4GD (for malate dehydrogenase), Procion Red H-3B (for 3-hydroxybutyrate dehydrogenase) and Procion Blue MX-R (for L-lactate dehydrogenase) are examples. These and others are summarised in the following table:

Group 1	Group 2	Group 3	Group 4	Group 5
P Blue MX-7RX	R Black GF	P Blue H-EG	P Black H-EXL	P Blue H-ERD
C Blue 2-RA	P Blue MX-R	P Blue H-EGN	P Blue H-GR	C Blue F-R
R Orange 3R	P Brown MX-GRN	P Blue H-4R	P Blue MX-G	P Brown H-5R
P Red MX-2B	C Brown 3-GRA	P Blue MX-3G	P Blue MX-4GD	P Green H-4G
P Rubine H-BN	P Navy H-4R	C Blue F3-GA	D Blue K-BL	P Green H-E4BD
P Turquoise H-A	P Orange MX-G	R Blue B	P Brown H-3R	P Navy H-ER
P Turquoise MX-G	R Orange FR	R Blue R	P Brown MX-5BR	P Red H-3B
C Turquoise 6-GE	P Red MX-5B	C Navy F-2R	P Orange H-ER	P Red H-8BN
R Violet R	P Scarlet MX-G	P Red H-E3B	P Orange MX-2R	P Red H-E7B
R Yellow GNL	P Scarlet MX-3G	P Rubine MX-B	P Red MX-7B	P Scarlet H-E3G
P Yellow H-A	C Turquoise GFP	P Scarlet H-2G	P Red MX-8B	P Yellow H-E3G
P Yellow MX-6G	C Yellow R-A	P Yellow H-E6R	C Red 3-BA	P Yellow H-E6G
P Yellow MX-8G	P Yellow MX-3G	P Yellow H-5G	P Violet H-3R	P Yellow H-E4R
	P Yellow MX-4R	P Yellow MX-R	P Yellow H-E6R	P Yellow MX-GR
		C Yellow 3-GP		

Group 1 dyes bind the least protein from crude extracts of tissues, and group 5 dyes the most. Actual groups may vary ± 1 with different types of extract. P, I.C.I. Procion; C, Ciba-Geigy Cibacron; R, Hoechst Remazol; D, Sandoz Drimarene. Not all of these dyes are still commercially available.

Source: From *J. Chromatogr.* **376**, 131-140 (1986)

The dye itself (with or without the spacer which is commonly used to attach the dye to a column) may cause the contamination, or the problem may be caused by a derivative of the dye or an intermediate used in the synthesis of the dye.

Cation-exchangers include S and CM Fast Flow, from Pharmacia.

Anion-exchangers include Pharmacia's DEAE Fast Flow and Q Fast Flow. Preferably, the matrix is Dowex-1, which is a strongly basic anion exchange resin, preferably 2% cross-linked, with a dry mesh size of 50-100. Generally, a strong anion exchanger is better than a weak exchanger.

The protein may be a serum-derived protein such as human albumin, a lipoprotein, a blood coagulation factor such as Factor VIII or Factor IX, thyroxin-binding globulin or alpha interferon. Preferably, the protein is human albumin (HA) or a mutant or fragment thereof which retains a dye-binding domain (such as is described in EP-A-322 094) or a fusion of HA or a said mutant or fragment with another protein. The aqueous liquid is suitably the direct or indirect result of exposing a fermentation medium or fractions thereof to the protein-binding compound; "indirect" in this context means that the fermentation medium, after contact with the protein-binding compound,

may be treated in one or more process steps before the process of the invention is applied. By "fermentation medium" we mean the medium which results from the fermentation of an organism capable of producing the protein. The organism (which term includes cell lines) is preferably transformed or transfected to produce the protein and the protein is normally heterologous to the organism. The organism may be a bacterium (eg *E. coli* or *B. subtilis*), a yeast (eg *Saccharomyces cerevisiae*), a non-yeast fungus (eg *Aspergillus niger*), an insect cell (eg *Spodoptera frugiperda*), a plant cell (eg a hairy root cell culture of *Atropa belladonna*) or a mammalian cell (eg Vero cells). Preferably, the organism is a yeast. Exemplary genera of yeast contemplated to be useful in the practice of the present invention are *Pichia, Saccharomyces, Kluyveromyces, Candida, Torulopsis, Hansenula, Schizosaccharomyces, Citeromyces, Pachysolen, Debaromyces, Metschunikowia, Rhodosporidium, Leucosporidium, Botryoascus, Sporidiobolus, Endomycopsis,* and the like. Preferred genera are those selected from the group consisting of *Pichia, Saccharomyces, Schizosaccharomyces, Kluyveromyces, Yarrowia* and *Hansenula*, because the ability to manipulate the DNA of these yeasts has, at present, been more highly developed than for the other genera mentioned above.

Examples of *Saccharomyces* are *Saccharomyces cerevisiae* (especially preferred), *Saccharomyces italicus* and

Saccharomyces rouxii. Examples of *Kluyveromyces* are *Kluyveromyces fragilis* and *Kluyveromyces lactis*. Examples of *Hansenula* are *Hansenula polymorpha*, *Hansenula anomala* and *Hansenula capsulata*. *Yarrowia lipolytica* is an example of a suitable *Yarrowia* species, and *Schizosaccharomyces pombe* is a further suitable yeast.

The production of human albumin expressed from a gene inserted into a suitable host by recombinant DNA techniques is well known in the art and does not require discussion herein. Examples of specific prior art processes include those described in EP-A-147 198 (Delta Biotechnology), EP-A-201 239 (Delta), EP-A-60 057 (Genentech), EP-A-88 632 (Genentech), EP-A-251 744 (Delta) and EP-A-286 424 (Delta).

Similarly, processes for purifying proteins from a fermentation medium are known in the art. A good review may be found in "Protein Purification - Principles and Practice", 2nd Edition (Springer Verlag, N.Y.), especially pages 141-157.

Preferably, the aqueous liquid results from passing the fermentation medium through one or more separation (eg chromatographic) steps.

It is to be noted that, although the process of the invention is particularly well suited to separating a protein-binding compound from a protein when the protein-

binding compound has been used to purify the protein from, for example, a fermentation medium or a product thereof, the process can generally be used to separate any suitable protein-binding contaminant from a protein. An advantage of the process is that it does not require binding of the protein to the resin and hence relatively large volumes of protein can be purified for a given volume of resin.

The disrupting material may be a single compound or a mixture. Preferably, it comprises a mixture of a salt (preferably sodium chloride or potassium chloride) and a compound to disrupt hydrophobic interactions between the protein and the protein-binding compound, for example a (preferably non-ionic) detergent, an organic solvent or, preferably, a fatty acid. Alternative disrupters of hydrophobic interactions with the protein include N-acetyltryptophan and mandelic acid, which will normally be used as their salts, for example sodium salts. The fatty acid is preferably octanoic acid but other fatty acids (preferably C_6-C_{10} and preferably saturated) may be used. The fatty acid will usually be present in the form of its salt, for example the sodium salt. The concentrates of the salt and fatty acid may be varied to suit the particular protein and protein-binding compound in question. A salt concentration of 0.1 M to 3 M will generally be useful, preferably 0.5 to 2.0 M. A fatty acid concentration of 10 mM-100 mM is generally useful, preferably 25-60 mM, most preferably about 50 mM. When the disrupting material is a

single compound, any of these materials may be used.

The liquid which is exposed to the ion exchange resin will usually consist largely of the buffer used to elute the protein from the column containing the protein-binding compound. The disrupting material or a component of it may then be added. For example, if the elution buffer contains 2 M NaCl in a 50 mM phosphate buffer of pH7.0, there may be no need to add further salt, and only the fatty acid is added. The pH can be altered if desired. We have found that a pH of about 7.0 is suitable, but generally any pH of above 5.0 is applicable to any fatty acid.

The pH should preferably be such that the protein-binding compound is charged; for example most polysulphonated triazine dyes are negatively charged above pH 2 to 3. It is not always necessary for the liquid to contain a buffer.

The most convenient means of exposing the mixture of the protein and protein-binding compound to the ion exchange resin and disrupting material will be to add the disrupting material to the mixture and then to pass the resulting liquid through a column of the ion exchange resin. This minimises the amounts of buffer and resin used, and the amount of protein lost. However, it is technically possible to expose the protein/protein binding compound mixture to the resin first, and then to elute the protein with a buffer containing the disrupting material. A larger

column of resin will usually be needed in such an embodiment, which will then probably have to be cleaned stringently with suitable acids and solvents rather than being simply discarded.

The columns may be the conventional linear type or radial flow cartridges.

The invention will now be illustrated by way of example and with reference to Figure 1 which shows the structure of a textile dye (Cibacron Blue 3-GA) and spacer (4-amino butyl group) usable in a column to purify human albumin.

Example 1

As a model of the product of passing an HA-containing fermentation medium through a purification column, a 3 $mg.ml^{-1}$ solution of human serum albumin was prepared in 2 M NaCl, 50 mM phosphate buffer pH7.0, and 21 $\mu g.ml^{-1}$ of Cibacron Blue dye covalently attached to a spacer (Fig 1) was added. The dye included a spacer used to attach the dye molecule to the matrix and also a dye synthesis intermediate. 1 M sodium octanoate, as the disrupter of hydrophobic interactions, was added to give a concentration of 50 mM. This solution (20 ml) was then passed through a 1 ml column of Dowex-1 resin (2% cross-linked; Dow Chemical Co) at a flow rate of 0.5 $ml.min^{-1}$. The removal of blue dye from HA was measured spectrophotometrically at 620 nm.

Under these conditions, about 97% of the blue dye bound to the resin. The unbound fraction which had passed through the column contained greater than 97% of the HA applied to the column.

Example 2

Following the procedure of Example 1, the efficiency of dye removal from HA was assessed in the presence of buffer, 2 M NaCl, caprylate and combinations of these components. As can be seen from the results in Table 1, a combination of salt and fatty acid was much more effective than the individual components.

TABLE 1

Buffer	Dye + Spacer Removal (%)	Dye Intermediate Removal (%)
A 50 mM phosphate pH7.0	19	N/D
B 50 mM phosphate + 2 M NaCl	32	N/D
C 50 mM phosphate + 50 mM caprylate	46	N/D
D Combination (B + C)	97	96

N/D = not determined

Example 3

The comparison of Example 2 was repeated, using Cibacron Blue 3-GA (Blue), Procion Green H-4G (Green), Procion Brown MX-5BR (Brown) and Procion Red HE-3B (Red) dyes covalently attached to a spacer. The results are shown in Table 2.

TABLE 2

	HSA/Dye + Spacer Separation (% removal)			
Buffer	Blue	Green	Brown	Red
A	19	11	52	33
B	32	41	93	57
C	46	45	90	83
D	97	65	89	92

A-D as Table 1

Example 4

The experiment of Example 2 was repeated with different proteins. The results are shown in Table 3. Alkaline phosphatase was mixed with blue or red dyes.

TABLE 3

	Protein/Dye + Spacer Separation (% removal)					
Buffer	HSA	LACTOFERRIN	ADH	GK	AP/Blue	AP/Red
A	19	66	N/D	N/D	52	N/D
B	32	N/D	42	73	80	63
C	46	67	72	96	80	71
D	97	81	94	91	84	92

A-D as Table 1 GK = glycerokinase
ADH = alcohol dehydrogenase AP = alkaline phosphatase
N/D = not determined

CLAIMS

1. A process for removing some or all of a protein-binding compound from an aqueous liquid containing the protein-binding compound and a protein to which it can bind or is bound, the process comprising the steps of (1) exposing to a disrupting material to disrupt binding of the protein to the protein-binding material, (2) exposing the liquid to an ion exchange resin to bind the protein-binding material to the resin and (3) separating the resin from the liquid.

2. A process according to Claim 1 wherein the protein-binding compound is a synthetic textile dye or an intermediate or derivative thereof.

3. A process according to Claim 2 wherein the protein-binding compound is a triazine dye or an intermediate or derivative thereof.

4. A process according to any one of the preceding claims wherein the ion-exchange resin is a strongly basic anion exchange resin.

5. A process according to any one of the preceding claims wherein the protein is human albumin (HA) or a mutant or fragment thereof which retains a dye-binding domain or a fusion of HSA or a said mutant or

fragment with another protein.

6. A process according to any one of the preceding claims wherein the disrupting material comprises a mixture of a salt and a compound to disrupt hydrophobic interactions between the protein and the protein-binding compound.

7. A process according to Claim 6 wherein the compound to disrupt hydrophobic interactions is a fatty acid or a salt thereof.

8. A process according to any one of the preceding claims wherein the mixture of the protein and protein-binding compound is admixed with the disrupting material and the resulting liquid is then passed through a column of the ion exchange resin.

9. A process according to any one of the preceding claims wherein the liquid is exposed to the disrupting material and then to the ion-exchange resin.

10. A process for preparing a protein comprising:-

(i) fermenting an organism capable of producing the protein such that the protein is produced,

(ii) exposing the protein-containing fermentation

medium obtained from step (i), or a liquid derived therefrom and containing the said protein, to an immobilised protein-binding compound,

(iii) separating the protein from the immobilised protein-binding compound, and

(iv) subjecting the protein from step (iii) to a process according to any one of Claims 1 to 8 to remove at least some of any protein-binding compound associated with the protein.

11. A protein obtained by a process according to any one of the preceding claims.

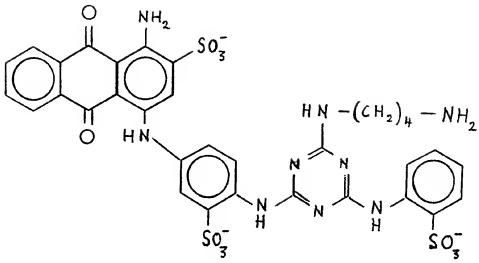

FIGURE 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01556

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 K 3/20 C 07 K 15/06

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	GB,A,2053926 (ATKINSON et al.) 11 February 1981, see pages 11-12	11
Y		1-10
X	DE,A,2342058 (ZENTRALNY ORDENA LENINA INSTITUT GEMATOLOGII I PERELIWANIJA KROWI) 27 February 1975, see pages 4,6	11
Y		1-10
Y	R.K. SCOPES: "Protein Purification", 2nd edition, 1988, pages 141-156, Springer-Verlag, (New York, US), see pages 155-156 (cited in the application) -/-	1-10

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05-12-1991	17. 01. 92
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: Mme. M. van der Drift

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	JORNAL OF CHROMATOGRAPHY, vol. 57, 1971, (Amsterdam, NL), M.-P. VAN DAMME et al.: "Séparation des stéroïdes et des colorants marqueurs sur résine échangeuse d'ions", paes 158-160, see page 158	1-10
A	TRANSFUSION, vol. 27, 1987, M.-J. KING: "Hemagglutination enhancement by bovine serum albumin is affected by octanoate, reactive blue 2 (Cibacron Blue), and polymer", pages 302-308, see page 306, column 2	1-11
A	CHEMICAL ABSTRACTS, vol. 95, 1981, page 205, abstract no. 127700v, (Columbus, Ohio, US), U. KRAGH-HANSEN: "Effects of aliphatic fatty acids on the binding of Phenol Red to human serum albumin", & BIOCHEM. J. 1981, 195(3), 603-13, see abstract	1-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. GB 9101556
SA 51155

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 07/01/92
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A- 2053926	11-02-81	None	
DE-A- 2342058	27-02-75	None	